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Synthesis of 2(S)-benzyl-3-[hydroxy(1'(R)-amino ethyl)phosphinyl]propanoyl-L-3-[^{125}I]-iodotyrosine : A Radiolabelled Inhibitor of Aminopeptidase N

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SUMMARY

2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl)phosphinyl]propanoyl-L-3-[^{125}I]-iodo tyrosine was prepared from 1(R)-(N-benzyloxycarbonylamino)ethylphosphinic acid in a six step synthesis. This new iodinated compound, which is a highly efficient aminopeptidase N inhibitor (Ki = 0.95 nM), can be used for complete characterization of the biochemical and pharmacological properties of aminopeptidase N and its *in vivo* inhibition. A high radiochemical purity was obtained with a specific activity of 2.17 Ci/mmol at the end of the synthesis.

Key words. Phosphinic compound / ^{125}I / Aminopeptidase N / Inhibitor.

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INTRODUCTION

Aminopeptidase N (APN, EC 3.4.11.2) is a membrane-bound zinc exopeptidase with a wide distribution in many tissues. It is located with highest levels in intestinal and kidney brush border membranes (1) but also found in the brain, the lung, blood vessels and primary cultures of fibroblasts (2). This enzyme is involved in the enzymatic cascade of the renin-angiotensin system by cleaving angiotensin III (3). In the brain, it could participate in the degradation of nociceptin (4) and behaves, in association with neutral endopeptidase (NEP, E.C.3.4.24.11), as one of the two ectopeptidases involved in the inactivation of the physiological opioid receptor ligands, enkephalins (5). Dual inhibitors of both enzymes APN and NEP have been shown to completely block the *in vivo* metabolism of enkephalins leading to strong spinal and supra-spinal analgesic effects (6-8).

The distribution of NEP has been well documented in the rat and pig, in both the central nervous system and the periphery (review in 5), demonstrating its co-localisation with μ and δ opioid receptors, but little is known concerning the distribution of APN except immunohistochemical studies performed in rat brain and periphery with an antiserum specific for rat APN (9-11). This method has shown that APN is localized on blood vessels in the brain and on proximal tubules in the kidney. The knowledge of the precise distribution of APN is crucial to investigate in depth its physiological role in different neuropeptide catabolism and for *in vivo* competition experiments with systemically administered inhibitors.

Due to their small size and their rapid diffusibility, radioactive inhibitors have the advantage over antibodies to interact quantitatively with the enzyme active site in all tissues and species. Moreover, the use of ^{125}I needs only short exposure times for autoradiograms, has a high sensibility and offers the possibility of a precise visualization of the enzyme localization.

We have recently described higher potent and selective APN inhibitors belonging to the series of α-aminophosphinic compounds (12,13). Therefore, using this approach and with the aim to explore the regional distribution of APN in the central nervous system and peripheral organs, we have developed the first highly selective and potent ^{125}I radiolabelled APN inhibitor, 2(S)-benzyl-3-3-[hydroxy(1'(R)-amino ethyl)phosphinyl]propanoyl-L-3-[^{125}I]-iodotyrosine (Figure

1). This molecule, which has a Ki of 0.95 nM, is an excellent ligand for binding studies and autoradiography and allows the direct detection of nanogram quantities of the enzyme from tissue extracts.

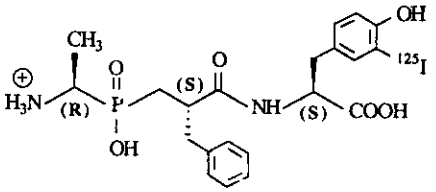

Figure 1. Structure of 2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl) phosphinyl]propanoyl-L-3-[^{125}I]-iodotyrosine

RESULTS AND DISCUSSION

A straightforward approach to the preparation of 2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl)phosphinyl]propanoyl-L-3-[^{125}I]-iodotyrosine (Figure 2) consists in starting from the precursor **5** and using the chloramine-T-mediated iodination.

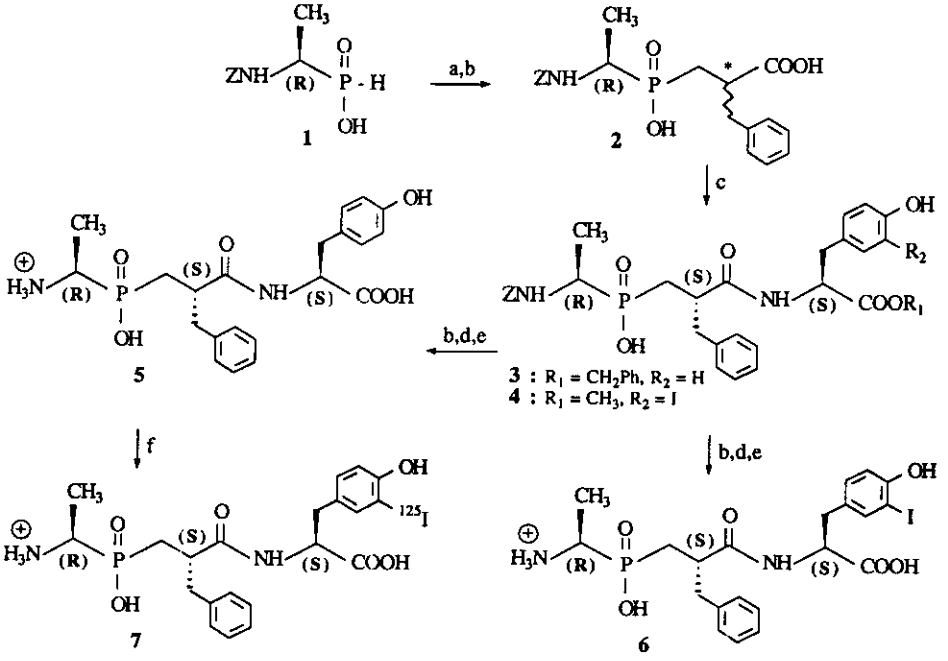

Figure 2. Synthetic scheme for the unlabelled standard 6 and the ^{125}I labelled inhibitor 7.
a : CH_2=C(CH_2Ph)CO_2CH_3/BSA ; b : 1N NaOH ; c : amino acids/EDCI/HOBt/DIEA ; d : HBr ; e : separation of the two diastereoisomers by semi-preparative HPLC ; f : Na^{125}I/chloramine-T.

The benzyloxycarbonyl (Z) protected 1(R)-(aminoethyl)phosphinic acid **1** was synthesized as previously described by Baylis (14). This compound was converted to the diastereoisomeric mixture of phosphinic acid **2** by condensation with methyl 2-benzylpropenoate in the presence of N,O-bistrimethylsilylacetamide (BSA), followed by alkaline hydrolysis. A coupling step with a C-protected tyrosine or 3-iodotyrosine and the sequential saponification of the carboxylic ester and acid cleavage of the protective group Z, led to the precursor **5** or unlabelled standard inhibitor **6**, as a mixture of two stereoisomers. These two diastereoisomers were easily separated by HPLC under semi-preparative conditions. The stereochemical assignment of **5** and **6** (R,S,S) was determined by ^{1}H NMR spectrocopy (15).

Radioiodination of 2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl)phosphinyl]-propanoyl-L-tyrosine **5** was performed by using 5 mCi of $Na^{125}I$ in aqueous sodium hydroxide solution with chloramine-T in 50 mM phosphate buffer, at pH 7.0. The reaction mixture was stirred for 15 minutes and then stopped by addition of 5 eq of $Na_2S_2O_5$. About 70% of radio-iodinated products correspond to the mono substituted inhibitor whilst the remaining compound was identified as the bis-iodinated inhibitor. The radiolabelled compound **7** obtained was purified by HPLC to provide 30% yield. The radioligand co-eluted with the unlabelled iodinated standard when a spiked aliquot of the purified product was analyzed by HPLC, while checking the radioactivity of collected fractions (Figure 3).

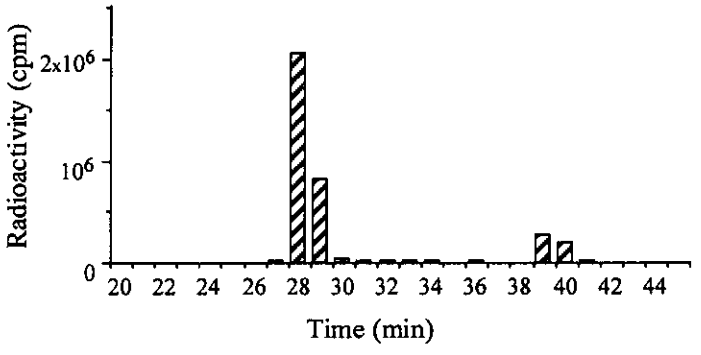

Figure 3. Radioactivity of compound 7 (the peak, centered at 39-40 min, corresponds to the bis-iodinated derivative of **6**).

In conclusion, 2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl)phosphinyl]-propanoyl -L-3-iodotyrosine **6**, a new iodinated derivative of phosphinic compounds, was

synthesized and evaluated *in vitro* for inhibition of pig kidney APN with L-alanine-p-nitroanilide (Ala-PNA) as substrate. This compound was found to have high affinity (Ki = 0.95 nM) towards APN and good selectivity versus other aminopeptidases. A selectivity factor of 200 was obtained versus APA and higher than 10,000 versus APB. The corresponding ^{125}I labelled inhibitor 7 was prepared in good yield by using the oxidative chloramine-T method. This new radiolabelled compound should provide a useful tool for distribution studies of APN in brain and periphery, and for in vivo studies of the specific or dual inhibitor bioavailabilities. Such studies are now in progress in our laboratory.

EXPERIMENTAL

The natural amino acid derivatives were purchased from Bachem (Bubbendorf, Switzerland). Reagents were from Aldrich (Strasbourg, France). $Na^{125}I$ was from Amersham. The solvents were from SDS (Peypin, France). TLC were revealed with UV, iodine vapor, or ninhydrin..The purity of the final compounds was checked by HPLC on a reverse phase chromasil C_8 (5 mm, 100 Å) column with 0.05% TFA in H_2O (solvent A)/CH_3CN (solvent B), as the mobile phase, on a Shimadzu apparatus. The eluted peaks were monitored at 210 nm.

The structure of all compounds was confirmed by ^1H NMR spectroscopy (Brüker AC 270 MHz) in DMSO-d_6 using HMDS as internal reference. Melting points of the compounds were determined on an Electrothermal apparatus and are reported uncorrected.

Methyl 2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonylamino)ethyl] phosphinyl}propanoate.

A solution of 1(R)-(N-benzyloxycarbonylamino)ethylphosphinic acid **1** prepared as described (11) (1 g, 4.12 mmol) and methyl 2-benzylpropenoate (0.87 g, 4.94 mmol) in 2.1 ml of N,O-bistrimethysilylacetamide were stirred overnight at 70° C. After cooling, the mixture was treated with water and extracted with ethyl acetate. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was triturated in hexane to give 1.59 g of product (92.2%) which was used for the following step without further

purification. mp 154°-155°C ; HPLC (45% B) 9.1 min ; NMR ^{1}H (DMSO d_6 and TFA) : 1.22 (dd, 3H, CH_3ß) ; 1.6-2.0 (m, 2H, P-CH_2) ; 2.65-3.0 (m, 3H, CH_2Ph, $CHCO_2$) ; 3.45 (s, 3H, CO_2CH_3) ; 3.7 (m, 1H, NCHa) ; 5.0 (s, 2H, CH_2(Z)) ; 7.0-7.38 (m, 10H, Ar) ; 7.46 (d, 1H, ZNH).

2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonylamino)ethyl]phosphinyl} propanoic acid (2)

The methyl 2(R,S)-benzyl-3-(hydroxy(1'(R)-(N-benzyloxycarbonylaminoethyl)phosphinyl)-propanoate (1 g, 2.39 mmol) was dissolved in 12 ml of methanol, and 12 ml of 1N NaOH (12 mmol) was added. The mixture was stirred for 16 h at room temperature. After acidification with 2N HCl, the methanolic layer was evaporated. The residue was taken up in water, and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over Na_2SO_4 and evaporated *in vacuo*, providing 0.88 g of product (90.5%). mp 175°-178°C ; HPLC (65% B) 4.2 min ; NMR ^{1}H (DMSO d_6) : 1.05-1.2 (dd, 3H, CH_3ß) ; 1.55-2.0 (m, 2H, P-CH_2) ; 2.7-2.95 (m, 3H, CH_2Ph, $CHCO_2$) ; 3.68 (m, 1H, NCHa) ; 4.48 (s, 2H, CH_2(Z)) ; 7.05-7.35 (m, 10H, Ar) ; 7.45 (d, 1H, ZNH).

2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonylamino)ethyl]phosphinyl} propanoyl-L-tyrosine Benzyl Ester (3)

To a solution of 2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonyl-amino)ethyl]phosphinyl}propanoic acid **2** (200 mg, 0.49 mmol) and 66 mg (0.49 mmol) of diisopropylethylamine in 2 ml of CH_2Cl_2 were added the tosylate of Tyr-OCH_2Ph (241 mg, 0.54 mmol) and 132 mg of diisopropylethylamine (0.98 mmol) in 2 ml of CH_2Cl_2, HOBT (66.5 mg, 0.49 mmol) in 2 ml of tetrahydrofuran and EDC (710 mg, 2.39 mmol) in 2 ml of CH_2Cl_2. After stirring for 45 min at room temperature, the mixture was filtered, and the filtrate was concentrated to an oily residue, which was taken up in ethyl acetate, filtered and concentrated. The residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH/AcOH (9/1/0.5) as eluent to give 205 mg of product (63.1%). mp 120-122°C ; HPLC (55% B) 6.6 and 6.9 min ; NMR ^{1}H (DMSO d_6) : 1.05-1.15 (dd, 3H, CH_3ß) ; 1.5-1.85 (m, 2H, P-CH_2) ; 2.55-2.95 (m, 5H, CH_2Ph, CH_2PhOH, $CHCO_2$) ; 3.65 (m, 1H, NCHa) ; 4.32 (m, 1H, CHCO) ; 4.95 (m, 4H, CH_2(Z), CO_2CH_2Ph) ; 6.5-7.4 (m, 20H, Ar, CONH) ; 8.38 (s, 1H, OH) ; 9.16 (d, 1H, ZNH).

2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonylamino)ethyl]phosphinyl} propanoyl-L-tyrosine Methyl Ester (4)

The reaction conditions were the same as described above, from 200 mg (0.49 mmol) of **2**, 247 mg of product (70.6%) was obtained. mp 113-115°C ; HPLC (50% B) 6.4 min ; NMR ^{1}H (DMSO d_6 and TFA) : 1.0-1.25 (dd, 3H, CH_3ß) ; 1.4-2.0 (m, 2H, P-CH_2) ; 2.55-3.0 (m, 5H, CH_2Ph, CH_2Ar, $CHCO_2$) ; 3.-3.55 (d, 3H, CH_3) ; 3.7 (m, 1H, NCHa) ; 4.28 (m, 1H, CHCO) ; 4.95 (s, 2H, CH_2(Z)) ; 6.5-7.4 (m, 14H, Ar, CONH) ; 8.2-8.4 (d, 1H, ZNH).

2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonylamino)ethyl]phosphinyl} propanoyl-L-tyrosine

To a solution of 2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonyl-amino)ethyl]phosphinyl}propanoyl-L-tyrosine benzyl ester **3** (180 mg, 0.27 mmol) in 1.6 ml of methanol was added 1.6 ml of 1N NaOH (1.6 mmol). The mixture was stirred for 8 h at room temperature. After acidification with 2N HCl, the methanolic layers were evaporated, diluted in water. The product was filtered and dried to provide 140 mg of product (90.2%). mp 178-180°C ; HPLC (40% B) 8.1 and 9.5 min ; NMR ^{1}H (DMSO d_6) : 1.05-1.18 (dd, 3H, CH_3ß) ; 1.48-1.88 (m, 2H, P-CH_2) ; 2.55-2.95 (m, 5H, CH_2Ph, CH_2PhOH, $CHCO_2$) ; 3.75 (m, 1H, NCHa) ; 4.25 (m, 1H, CHCO) ; 4.98 (s, 2H, CH_2(Z)) ; 6.5-7.3 (m, 19H, Ar) ; 7.38 (d, 1H, CONH) ; 8.18 (d, 1H, ZNH) ; 9.1 (s, 1H, OH).

2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonylamino)ethyl]phosphinyl} propanoyl-L-3-iodotyrosine

The reaction conditions were the same as described above, from 240 mg (0.34 mmol) of **4**, 214 mg of product (91%) was obtained. mp 171-173°C ; HPLC (45% B) 7.1 and 8.3 min ; NMR ^{1}H (DMSO d_6) : 1.05-1.2 (dd, 3H, CH_3ß) ; 1.4-1.9 (m, 2H, P-CH_2) ; 2.5-2.95 (m, 5H, CH_2Ph, CH_2PhOH, $CHCO_2$) ; 3.75 (m, 1H, NCHa) ; 4.25 (m, 1H, CHCO) ; 4.98 (s, 2H, CH_2(Z)) ; 6.62-7.5 (m, 14H, Ar, CONH) ; 8.1-8.2 (dd, 1H, ZNH) ; 10.05 (d, 1H, OH).

2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl)phosphinyl]propanoyl-L-tyrosine (5)

2(R,S)-benzyl-3-{hydroxy[1'(R)-(N-benzyloxycarbonylamino)ethyl]phosphinyl}pro-panoyl-L-tyrosine (110 mg, 0.19 mmol) was dissolved in 2.5 ml of 33% HBr in acetic acid. The mixture was stirred at room temperature for 45 min. The solvents

were evaporated. The two stereoisomers were separated by HPLC using a semi-preparative column (HPLC, Kromasil C_8, 10 mm, 20x250 mm), to provide 55 mg of product with configuration (R,S,S) (51.8%) and 26.7 mg of product with configuration (R,R,S) (25.2%). mp 220°C (dec.) ; HPLC (20% B) 6.1 and 9.6 min ; NMR ^{1}H (DMSO d_6) : 1.08-1.2 (dd, 3H, CH_3ß) ; 1.4-1.8 (m, 2H, P-CH_2) ; 2.48-2.9 (m, 5H, CH_2Ph, CH_2PhOH, $CHCO_2$) ; 3.0 (m, 1H, NCHa) ; 4.24 (m, 1H, CHCO) ; 4.98 (s, 2H, CH_2(Z)) ; 6.5-7.2 (m, 9H, Ar) ; 7.8 (br. s, 3H, NH_3) ; 8.25 (d, 1H, CONH) ; 9.1 (s, 1H, OH).

2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl)phosphinyl]propanoyl-L-3-iodo-tyrosine (6)

The reaction conditions were the same as described above, from 124 mg (0.18 mmol) of **4**, 45 mg of 6 with configuration (R,S,S) (37.4%) and 35 mg with configuration (R,R,S) (29.1%) were obtained. mp 210°C (dec.) ; HPLC (30% B) 5.0 and 8.7 min ; NMR ^{1}H (DMSO d_6 and TFA) : 1.05-1.2 (dd, 3H, CH_3ß) ; 1.4-2.1 (m, 2H, P-CH_2) ; 2.55-3.0 (m, 6H, CH_2Ph, CH_2Ar, $CHCO_2$, NCHa) ; 4.3 (m, 1H, CHCO) ; 6.5-7.5 (m, 8H, Ar) ; 7.98 (br. s, 3H, NH_3) ; 8.35-8.5 (dd, 1H, CONH).

2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl)phosphinyl]propanoyl-L-3-[^{125}I]-iodo-tyrosine (7)

To a solution of sodium [^{125}I] iodide (5 mCi, 2.3 nmol) in aqueous sodium hydroxide solution were added, at 6°C, 2(S)-benzyl-3-[hydroxy(1'(R)-aminoethyl)phosphinyl]-propanoyl-L-tyrosine (**5**) (2.3 nmol) in 2.5 ml of 50 mM phosphate buffer, at pH 7.0 and 2.3 nmol of chloramine T in 6.4 ml of the same buffer. After standing for 15 min, the reaction was stopped by addition of 5 eq of $Na_2S_2O_5$. The desired radiolabelled inhibitor was purified by HPLC on a reverse phase Kromasil C_8 (5 mm, 100 A) column (mobile phase: 0.05% TFA in water/CH_3CN) eluted under gradient conditions for 18-28% CH_3CN at a flow rate of 1 ml/min in 20 min, to provide 1.5 mCi of product at a specific activity of 2.17 Ci/mmol.

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REFERENCES

1. Maroux S., Louvard D and Barrati J. *Biochim. Biophys. Acta.* **321:** 282-295 (1973).
2. Sanderink G.J., Artur Y., and Seist G.J. *Clin. Chem. Clin. Biochem.* **26:** 795-807 (1988).
3. Zini S., Fournié-Zaluski M.C., Chauvel E., Roques B.P., Corvol P., and Llorens-Cortes C. *Proc. Natl. Acad. Sci. USA* **93:** 11968-11973 (1996).
4. Montiel J.L., Cornille F. Roques B.P. and Noble F. *J. Neurochem.* **68:** 354-361 (1997).
5. Roques B.P., Noble F., Daugé V., Fournié-Zaluski M.C. and Beaumont A. *Pharmacol. Rev.* **45:** 87-146 (1993).
6. Bourgoin S., Le Bars D., Artaud F., Clot A.M., Bouboutou R., Fournié-Zaluski M.C., Roques B.P., Hamon M. and Cesselin F. *J. Pharmacol. Exp. Ther.* **238:** 360-366 (1986).
7. Dickenson A.H., Sullivan A.F. and Roques B.P. *Eur. J. Pharmacol.* **148:** 437-439 (1988).
8. Chen H., Noble F., Coric P., Fournié-Zaluski M.C. and Roques B.P. *Proc. Natl. Acad. Sci. USA* **95:** 12028-12033 (1998).
9. Turner A.J., Hooper N.M. and Kenny A.J. In : Kenny A.J. and Turner A.J., Editors. *Mammalian Ectoenzymes*. Elsevier Science Publishers B.V., 1987, pp. 211-248.
10. Hersh L.B., Aboukhair N. and Watson S. *Peptides* **8:** 523-532 (1987).
11. Solhonne B., Gros C., Polard H. and Schwartz J.C. *Neuroscience* **22:**225-232 (1987).
12. Chen H., Noble F., Coric P., Fournié-Zaluski M.C. and Roques B.P. *Proc. Natl. Acad. Sci.* **95(20):**12028-12033 (1998).
13. Chen H., Roques B.P. and Fournié-Zaluski M.C. *Bioorg. Med. Chem. Lett.* (1999), in press.
14. Baylis E.K., Campbell C.D. and Dingwall J.G. *J. Chem. Soc.* 2845-2853 (1984).
15. Fournié-Zaluski M.C., Lucas-Soroca E., Devin J. and Roques B.P. *J. Med. Chem.* **29:** 751-757 (1986).

